derivatives. These range from indirect methods such as the formation of diastereoisomeric derivatives or direct methods that exploit the spatial characteristics of different enantiomers by making them interact with a chiral stationary or mobile phase. This selectively enhances the standard free entropy of distribution of one amino acid enantiomer compared to the other and can provide adequate chiral selectivity to permit enantiomeric resolution. By one or other of these approaches the separation of the enantiomers of the majority of naturally occurring amino acids can be achieved by liquid chromatography.

See also: **II/Chromatography: Liquid:** Derivatization. **III/Chiral Separations:** Capillary Electrophoresis; Cellulose and Cellulose Derived Phases; Chiral Derivatization; Countercurrent Chromatography; Crystallization; Cyclodextrins and Other Inclusion Complexation Approaches; Gas Chromatography; Ion-Pair Chromatography; Ligand Exchange Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Supercritical Fluid Chromatography; Thin-Layer (Planar) Chromatography.

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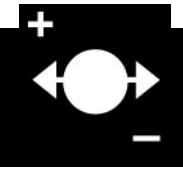
AMINO ACIDS AND PEPTIDES: CAPILLARY ELECTROPHORESIS



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Introduction

Advancement in modern biotechnology is mainly attributed to a detailed understanding of the structural features of proteins. This is predominantly accomplished by sequencing techniques and the analysis of amino acid composition. Irregularities in the structural characteristics of proteins, e.g. after translation of the protein, are determined by fragmentation to smaller peptides. Progress in the field of synthetic peptides utilizing synthesis based on these partial sequences depends on their immunological potential. The design of new specialized biomolecules such as hormones or neurotransmitters will have considerable pharmaceutical applications.

The large number of analytes and the small quantities present in biological samples have increased the demand for specific and sensitive analytical techniques. Capillary electrophoresis (CE) is capable of handling small sample volumes down to microlitre size with only a few nanolitres injected. High efficiencies, short analysis time and easy enantiomeric assays make CE an indispensable tool in the modern analysis of peptides and amino acids.

Amino Acids

Physicochemical Properties

In choosing an electrophoretic system it is important to consider both matrix and the structural features of the analytes. Whereas 18 amino acids are found after the hydrolysis of proteins, more than 50 derivatives are present in physiological fluids.

Amino acids are small, highly polar species. The individual species only differ in the residues *R*. Except for glycine this situation induces a chiral centre at the α-C-atom where two enantiomers (*R*-, *S*-) can be distinguished. Classifying these residues *R* by their impact on electrophoretic behaviour means a differentiation by their polarity or the generation of charge. Due to their zwitterionic nature, amino acids possess isoelectric points (pI); pH values equal to pI yield molecules without net charge and therefore no migration occurs in an electrical field. At pH values above the pI the molecules are negatively charged and migrate against the electroosmotic flow (EOF) towards the anode, whereas lower pH values induce cations which migrate with the EOF towards the cathode.

Most amino acids lack suitable physical characteristics that can be exploited for detection. Only few species possess aromatic groups with high absorptivity, e.g. try, phe and tyr. In order to analyse native amino acids three strategies can be pursued. UV detection can be used at low wavelengths. A second approach is the application of indirect detection techniques. Detection concepts involving derivatization technology, especially fluorescence labelling, can also improve detection sensitivity.

Electrophoretic Systems – Separation Strategies

Analysis of native amino acids Direct UV detection at wavelengths below 220 nm takes advantage of the absorptivity of the carbonyl bond. Detection at such nonspecific wavelengths requires highly transparent buffers. Borate and phosphate are convenient electrolyte systems. Selectivity is mainly achieved by the optimization of pH because the analysis is performed with the native species.

In order to obtain cationic analytes, pH has to be adjusted to values lower than the first dissociation step ($pK \sim 2$). The stability of fused-silica capillaries is restricted to pH values above 2.5. Thus basic conditions with analytes migrating counter to the EOF are preferred. This separation mode benefits by prolonging the effective separation distance, keeping the electrical field strength constant so that higher resolution is achieved. Limits of detection are in the range of about 10^{-4} mol L^{-1} (**Figure 1**).

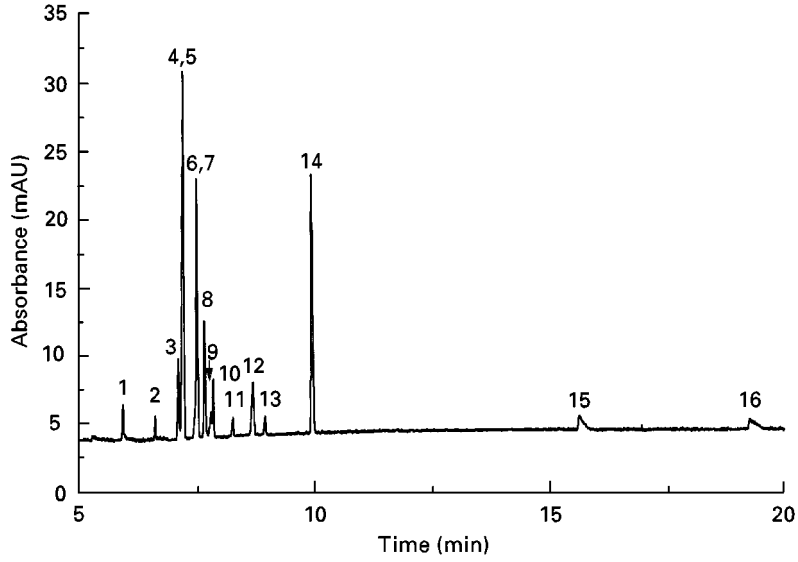

Figure 1 Separation of amino acids and dipeptides in an infusion solution using direct detection at low wavelength. Capillary: fused silica 75 μm i.d., 65/73.5 cm; buffer: borate 40 mmol L^{-1}, pH = 11.0; E = 408 V cm^{-1}, 191 nm; injection 50 mbar, 5 s. 1, Lys; 2, Pro; 3, Try; 4, Leu; 5, Ile; 6, Gly-Glu; 7, Val; 8, Phe; 9, His; 10, Met; 11, Ala; 12, Thr; 13, Ser; 14, Gly-Tyr; 15, Glu; 16, Asp.

Indirect UV detection was evolved for the analysis of small inorganic ions but it is also an efficient technique for analysis of a broad range of nonabsorbing components. This methodology is performed very easily with CE using a UV-absorbing electrolyte. With respect to dissociation behaviour, mobility and absorptivity the background electrolyte (BGE) has to be chosen carefully. As mentioned above, basic conditions should be applied to generate anionic species of amino acids. Therefore the BGE has to be negatively charged under alkaline conditions. Beside generating the background signal the nature of the electrolyte used has great influence on separation selectivity. Best resolution can be achieved with electrolytes of moderate mobility, e.g. salicylic acid ($\mu_{pH=11.5} = -6 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$). Salicylate at low mmol L^{-1} concentrations may also be used for indirect fluorescence detection. Concentration limits are in the range of 10^{-5} mol L^{-1} (**Figure 2**).

Another approach to a universal, high sensitivity detection scheme is mass spectrometry (MS). Beside the very low limits of detection which are achievable, this technique provides information about molecular mass and structure. The compatibility of capillary zone electrophoresis (CZE) to MS can be attributed to the low flow rates in CZE. The main problem in coupling CZE to MS is the buffer. Further developments on suitable volatile buffers and interface types will extend the scope of applications.

Analysis of derivatized amino acids Many of the chemical reactions for labelling originate from peptide synthesis where they were used as protective groups or sequencing agents.

As a consequence of derivatization, amino acids change from small ionic species to large hydrophobic molecules. Differences in mobilities decrease. A sufficient separation selectivity is mainly achieved by micellar electrokinetic capillary chromatography (MEKC).

Many reagents have been investigated to improve sensitivity as well as suitability for fluorescence detection. Depending on the separation problem, further requirements have to be considered. The reagent must react quantitatively and reproducibly with primary and secondary amines to form stable products. Side reactions and fluorescence of the tag itself can interfere with the analysis. The choice of derivatizing agent is limited by these prerequisites.

The commonest applied systems are discussed below (**Figure 3**).

The classical agent ninhydrin is not used for derivatization in CE because the aldehydes formed cannot be separated.

O-phthaldialdehyde (OPA) was one of the first reagents developed for pre-column derivatization in liquid chromatography (LC). Strongly absorbing isoindoles with fluorescence properties are formed in a rapid reaction. The stability of the derivatives mainly depends on the amino acid and the reducing agent, e.g. thiols. Unfortunately, secondary amines are not derivatized. An increase in stability and detection sensitivity has been achieved by using naphthalene-2,3-dicarboxaldehyde (NDA) or 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA).

Phenylthiohydantoins (PTH) of amino acids are generated during Edman degradation of peptides. Maximum absorbance is found at 254 nm but the

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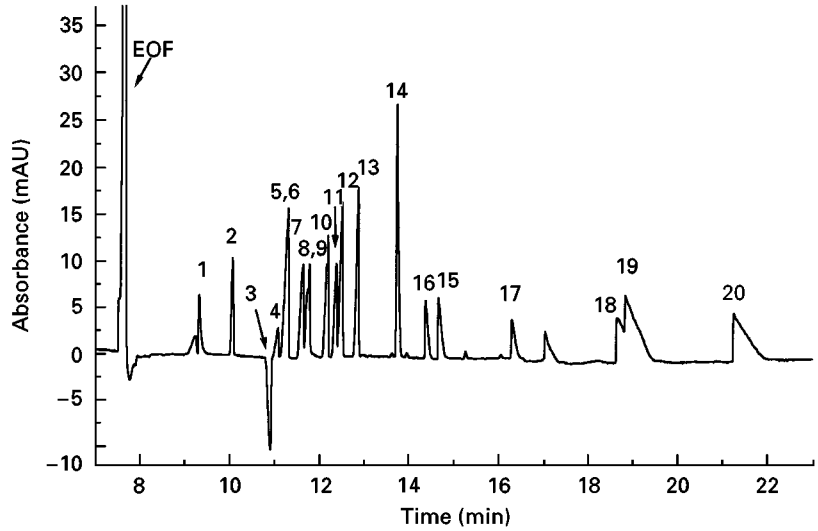

Figure 2 Separation of amino acids and dipeptides using indirect detection. Capillary: fused silica 75 μm i.d., 86.5/95 cm; buffer: salicylic acid 5 mmol L^{-1}; pH = 11.5; E = 316 V cm^{-1}, 214 nm; injection 50 mbar, 5 s. 1, Lys; 2, Pro; 3, Try; 4, Gly-Glu; 5, Leu; 6, Ile; 7, Val; 8, His; 9, Met; 10, Ala; 11, Thr; 12, Asn; 13, Ser; 14, Gly; 15, Tyr; 16, Ac-Tyr; 17, Cys-Cys; 18, Ac-Cys; 19, Glu; 20, Asp.

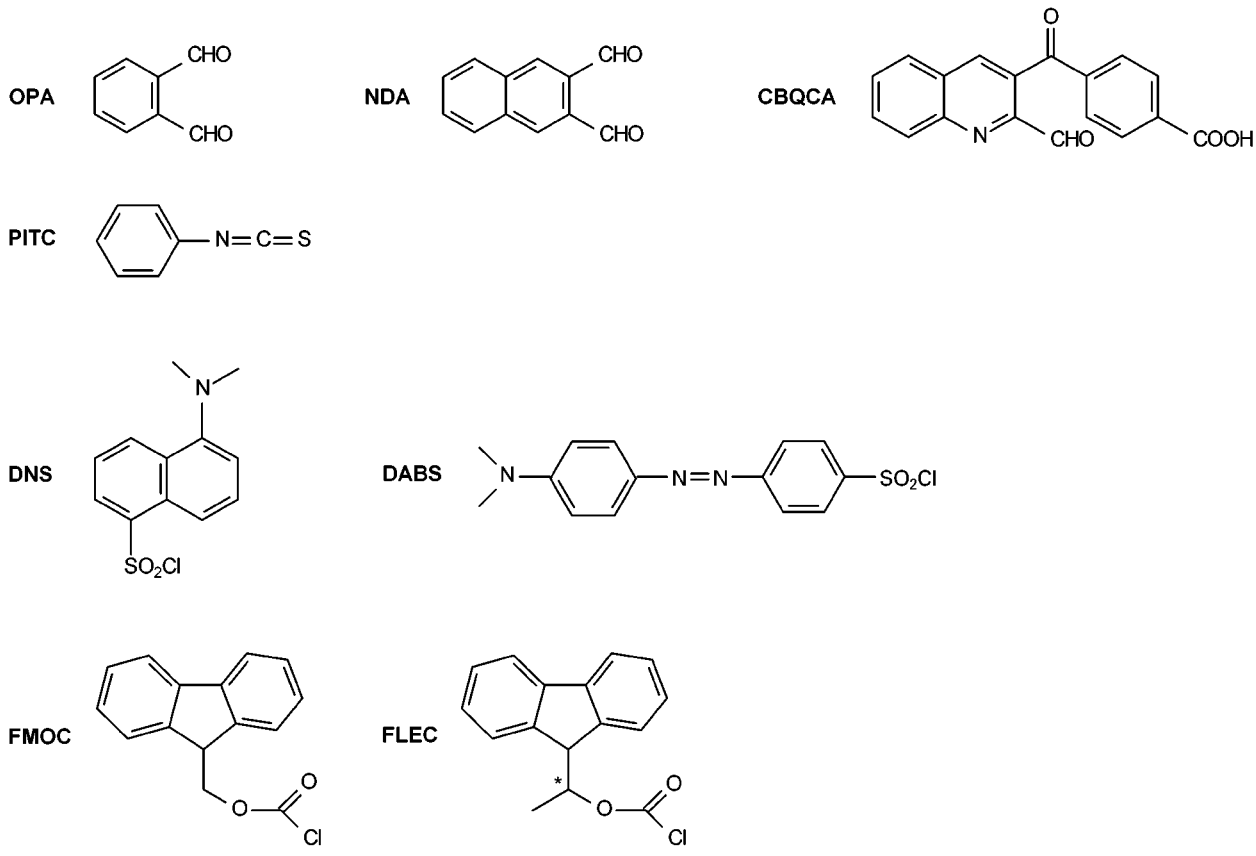

Figure 3 Structures of derivatizing reagents. OPA, *o*-Phthalaldehyde; NDA, naphthalene-2,3-dicarboxaldehyde; CBQCA, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde; PITC, phenylisothiocyanate, DNS, 5-dimethylaminonaphthalene-1-sulfonyl chloride; DABS, dimethylaminoazobenzenesulfonyl chloride; FMOC, 9-fluorenylmethyl chloroformate; FLEC, (*R*) (*S*)-1-(fluorenyl) ethyl chloroformate.

derivatives lack fluorescence. Analysis is performed using phosphate or borate buffers under alkaline conditions. Surfactants such as sodium dodecyl sulfate (SDS) give a micellar pseudo-stationary phase allowing the partition process. In contrast to cationic surfactants, e.g. dodecyltrimethylammonium bromide (DTAB), analytical systems using anionic surfactants benefit from a wider migration time window. This can be mainly attributed to their counterosmotic migration behaviour (**Figure 4**).

Sulfonyl chlorides can convert primary as well as secondary amines. Well-known representatives are dansyl (DNS) and dabsyl (DABS) chloride. In order to separate all DNS amino acids, acidic buffers are used to reduce the EOF. In addition, neutral surfactants such as TWEEN 20 have been applied. The main disadvantage is the prolonged analysis time of about 70 min. Faster separations can be achieved using SDS with the penalty of a decrease in resolution. In some cases resolution can be enhanced by operating at lower temperatures (**Figure 5**).

Carbonyl chlorides such as fluorenylmethyl chloroformate (FMOC) are more reactive than sulfonyl chlorides. FMOC amino acids fluoresce strongly and are stable at room temperature. Detection sensitivities in the nmol L^{-1} range can be achieved.

Beside fluorescence detection, further improvements in sensitivity and specificity can be obtained with laser-induced fluorescence (LIF) techniques. A prerequisite is the match of emission wavelengths of the derivatized analyte with the spectral lines of the lasers. Great effort has been invested in the development of new fluorophores such as TRTC, CTSP, TBQCA, IDA and CBQ (**Table 1**).

Unfortunately, most of them are not commercially available.

Different derivatization techniques are applied: precolumn tagging is the commonest method. Several attempts have been made to transfer post-column methodology from LC to CE. A further promising technique is derivatization in the capillary because it simplifies automation. Reagent and sample are injected in succession. With the tandem mode a plug of reagent is injected into the column followed by the sample. A second technique is the introduction of an additional plug of reagent after the sample (sandwich

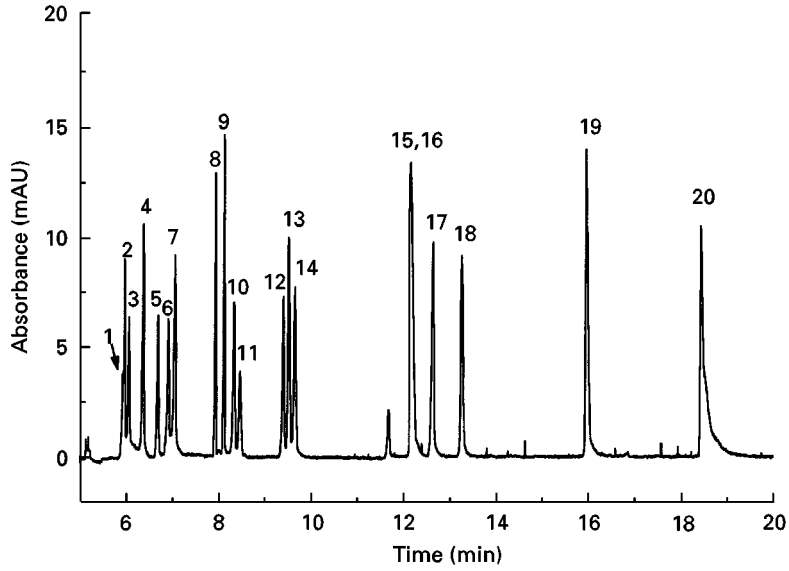

Figure 4 Separation of 20 PTH amino acids by MEKC. Capillary: fused silica 50 μm i.d., 59/67.5 cm, buffer: phosphate 25 mmol L^{-1}; SDS 25 mmol L^{-1}; pH = 9.0; E = 444 V cm^{-1}, 260 nm; injection 50 mbar, 5 s. 1, Thr; 2, Asn; 3, Ser; 4, Gln; 5, Asp; 6, Gly; 7, Ala; 8, His; 9, Glu; 10, Tyr; 11, Cys; 12, Pro; 13, Val; 14, Met; 15, Leu; 16, Ile; 17, Try; 18, Phe; 19, Lys; 20, Arg.

mode). After a specified time for reaction, the separation can be performed.

Chiral analysis Assays of enantiomeric purity are easily performed by CE by simply adding the chiral selector to the running buffer. Two different methodologies are applied to achieve resolution. First, chiral distinction can be established by the formation of non-covalently bonded diastereomers.

The most widely applied cyclodextrins form host–guest complexes with one of the enantiomers preferentially. Compared to migration in the bulk phase, the complexed species possesses a different mobility. The separation occurs due to different complex stabilities resulting in different migration velocities. Enhancement of enantioselectivity is primarily attributed to cavity size (α-, β-, γ-cyclodextrin (CD)) and derivatization of the hydroxy moieties of

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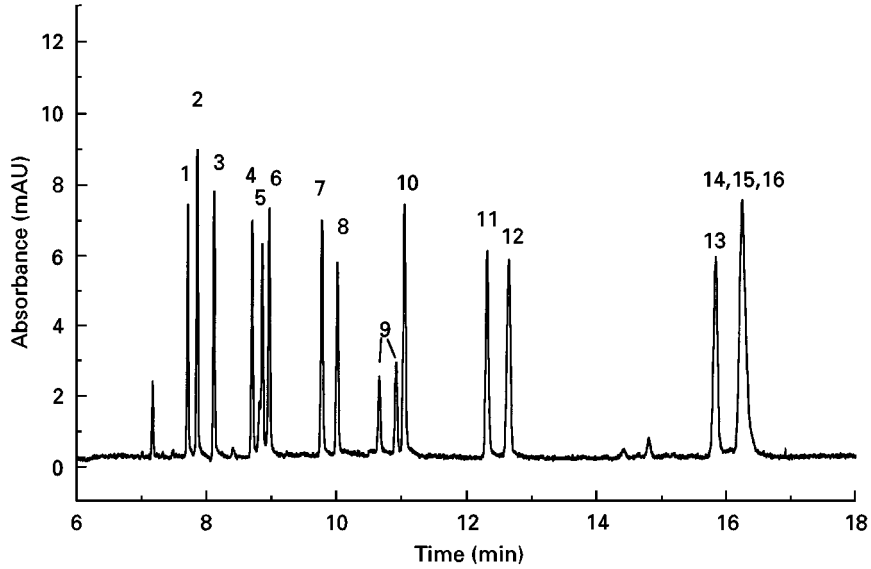

Figure 5 Separation of DNS amino acids by MEKC in an infusion solution. Capillary: fused silica 50 μm i.d., 50/57.5 cm, buffer: borax 20 mmol L^{-1} SDS 102.5 mmol L^{-1}; pH = 9.1; E = 435 V cm^{-1}, 214 nm; T = 7.5°C; injection 50 mbar, 5 s. 1, Thr; 2, Ser; 3, Ala; 4, Gly; 5, Glu; 6, Val; 7, Pro; 8, Met; 9, Ile; 10, Leu; 11, Phe; 12, Try; 13, Arg; 14, His; 15, Tyr; 16, Di D-Lys.

Table 1 Examples of derivatizing reagents and detection wavelengths

	ε (mol L^{-1} cm^{-1})	$\lambda_{abs/ex}$ (nm)	λ_{em} (nm)	Remark
OPA		334	455	Presence of reducing agents (thiols), strong absorbance, strongly fluorescence, unreacted OPA not fluorescent, derivatives lack of stability, reaction rapid
NDA		462	490	Reaction rapid, increased stability compared to OPA,
CBQCA		450		recently commercially available
PITC		254		Peptide sequencing by Edman degradation, cyclic thiohydantoins; no fluorescence properties
DNS	14 100	254	570	Problems with derivatization by-products
DABS		420-450		
FMOC-Cl		265	315	Fluorogenic derivatives with primary/secondary amines, strong absorbance
FLEC		265	310	Converts enantiomers to diastereomers
TRTC	$>$ 100 000	540	567	Ex at 540 nm matches with emission line of low cost HE laser
CTSP	82 000	663	687	Semiconductor laser
TBQCA		465	550	
IDA	33 100	409	482	
CBQ		466	544	

OPA, *o*-Phthalaldehyde; NDA, naphthalene-2,3-dicarboxaldehyde; CBQCA, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde; PITC, phenylisothiocyanate; DNS, 5-dimethylaminonaphthalene-1-sulfonyl chloride; DABS, dimethylaminoazobenzenesulfonyl chloride; FMOC, 9-fluorenylmethyl chloroformate; FLEC, (*R*) (*S*)-1-(-fluorenyl)ethyl chloroformate; TRTC, tetramethylrhodamine isothiocyanate; CTSP, pyronin succinimidyl ester; TBQCA, 3-(4-tetrazolebenzoyl)-2-quinolinecarboxaldehyde; IDA, 1-methoxycarbonylinodolizine-3,5-dicarbaldehyde; CBQ, 3-(*p*-carboxybenzoyl) quinoline-2-carboxaldehyde.

the cyclodextrin (methyl-, hydroxypropyl-, sulfobutyl-CD). Whereas compounds with a single aromatic core fit into α-CDs, β-CDs mainly form complexes with polynuclear aromates such as tyr or try. Larger structures are accommodated by γ-CDs. Most of the enantiomeric separations are performed using phosphate or borate electrolytes with native β- or γ-CD or mixed MEKC-CD systems which additionally contain a surfactant, mostly SDS.

Additives like urea or small amounts of organic solvents can improve the resolution.

Chiral surfactants such as *N*-dodecanoyl-L-serine (SDVal) or *N*-dodecanoyl-L-glutamate (SDGlu) have been investigated. These amino acids with hydrophobic alkyl chains are applied in a mixture with nonchiral surfactants, e.g. SDS.

Metal ions of copper(II), zinc(II) or cobalt(III) can be added to the electrolyte containing an L-isomer of an amino acid, e.g. L-proline, L-histidine or a dipeptide, e.g. aspartame. These metal–amino acid or metal–dipeptide complexes preferentially form a ternary complex with one enantiomer of the amino acid in the sample. Separation occurred due to different complex stabilities resulting in different mobilities for the individual enantiomers.

As a second approach, a racemic mixture of amino acids is derivatized with an optically pure reagent yielding covalent-bonded diastereoisomers. Reagents like GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate) allow the application of nonchiral separation techniques. Detection sensitivity can be improved simultaneously by using reagents like FLEC ((*R*) or (*S*)-(1-fluorenyl)ethyl chloroformate) containing chromophores or OPA with a chiral thiol.

Peptides

Peptides are compounds of great medical interest due to their physiological role as hormones and neurotransmitters. Furthermore, considering peptides as subunits of proteins, peptide mapping after chemical or enzymatical cleavage allows characterization of the protein and to reveal metabolic disorders.

Physicochemical Nature of Peptides

The characteristics of peptides are situated between those of amino acids and high molecular weight proteins. Oligopeptides containing up to 15 amino acids behave similarly to amino acids. Short peptide chains cannot create a complicated conformation. In contrast, very long polypeptides with chain lengths up to approximately 100 units behave like small proteins. They exhibit characteristic features of secondary and tertiary structure.

Peptides exist in aqueous solution as amphoteric ions. Therefore peptides possess isoelectric points (pI). The peptide has net electroneutral properties at

the pI. The zwitterionic characteristics are influenced predominantly by the acidity of the medium. In acidic media the carboxyl group ($pK_a \sim 2.7–2.9$) is protonated and the peptide behaves as a cation. In alkaline media the protonated ammonium group is eliminated and the zwitterionic form is converted into an anion. The degree of dissociation is determined by the dissociation constants of the functional groups yielding different net charges.

A further feature to be considered in electrophoretic behaviour is the sequence of the amino acids. The dissociation constants of the individual residues are affected by the arrangement of the amino acids in the chain. Mass-to-charge ratios are altered and the peptide exhibits a different mobility.

Prediction of Electrophoretic Mobility of Peptides

A theoretical model of electrophoretic migration underlying the experimental approach can be very useful for the optimization of analytical conditions. It supports predicting peptide mobilities under different experimental conditions such as pH. If selectivity between closely migrating species has to be implemented, the model facilitates adjustment of the separation environment.

Furthermore, considering technical processes and purity control of peptide synthesis or enzymatic digestion of proteins, a defined relationship between apparent mobility and physicochemical parameters supports the identification of unknown species. Variation in the sequence of peptides can also be easily determined.

The mathematical description of the migration process is based on the contribution of two forces. The electrical field accelerates an ion with a force proportional to its charge. In addition, the ion is influenced by a retarding force which results from the viscosity of the medium and is connected to a size parameter.

For permanently ionized small ions a prediction of migration is easily achieved by applying Stoke's law which correlates mobility with $q \times r^{-1}$ and $q \times M^{-1/3}$ (q, charge and M, molecular mass). With larger, more complex aggregates like peptides both the charge and a suitable size parameter has to be ascertained. For computing the charge, the sequence of amino acids has to be considered as the environment of a residue affects the extent of ionization, e.g. neighbouring amide bond or acidic/basic residues at terminal amino or carboxylic groups. This means that the ionization constants of the free amino acids have to be adjusted. During the development of a theoretical understanding of migration phenomena, many approaches have been made considering mass, surface, radius and the number of units in a peptide chain as size parameter.

The following equation results from the semi-empirical approach by Offord relating electrophoretic mobility μ of peptides with their charge q and their molecular mass M:

$$\mu = k \times q \times m^{-2/3}$$

This linear relationship has been validated by experimental research; a large set of analytes covering collagen fragments, tryptic digest of human growth hormone (33 peptides), motilin fragments (24 peptides) and many additional peptides differ widely in charge and amino acid sequence. Nowadays several computer programs are available which are capable of calculating the charge-to-mass ratio just requiring the amino acid sequence.

Electrophoretic Systems – Separation Strategies

To optimize the separation of peptides, the experimental conditions have to be adjusted to emphasize differences in the charge-to-mass ratios of the analytes.

Apart from external parameters like electrical field, capillary dimensions (length, inner diameter) and temperature, separation is mostly influenced by the electrolyte. Intrinsic variables like type of buffer, mobility, ionic strength, pH and buffer additives determine electrophoretic and electroosmotic mobility.

In the first place selectivity in the analysis of peptides is controlled by pH. Altering the acidity of the separation medium affects both the charge of the peptide and the ionization of the capillary wall, resulting in the change in EOF.

The hysteresis-like course of the EOF shows the greatest variation in the pH range of approximately 5–7, i.e. near the dissociation constant of the silanol groups. For pH values below 3 or greater than 9, the influence of the superimposed EOF can be neglected and the migration of the peptide is almost independent from the EOF.

In acidic media (pH $\sim$ 2) both basic and acidic residues of the peptide are protonated. Selectivity is attributed to the number of positive-charged ammonium groups in the chain resulting in different charge densities. Analytes migrate with the EOF. In high pH buffers (pH $\sim$ 10), deprotonation of terminal and side chain ammonium groups (His) induces negatively charged species (presence of carboxylate groups) which migrate in the opposite direction to the EOF. At higher pH values the side chain amino groups of arg and lys are the only ones affected.

Optimization of pH values below 2 and above 12 is difficult to achieve since the limiting values of mobility are reached. Furthermore, due to the high conductivities of protons and hydroxyl ions, high currents

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accompanied by Joule heating are generated. For practical purposes selectivity control for peptides with a majority of acidic moieties is mainly achieved in the range of pH 3–6 while basic residues are mostly affected at pHs around 10.

Additionally, isoelectric points of the peptides have to be included in the optimization strategy.

If peptides are obtained by chemical or enzymatic digests of proteins the cleaving agent has to be considered, e.g. trypsin cuts at the C-terminal side of lys and arg respectively. Thus fragments contain an excess of acidic residues. Selectivity can be easily affected in acidic media. Cleavage at aromatic or aliphatic side chains is performed with chymotrypsin or pepsin, yielding fragments with both acidic and basic residues and optimization can be extended to the full pH range (**Figure 6**).

Frequently used electrolytes for peptide mapping are phosphate, citrate and acetate as acidic buffers while borate or TRIS/Tricine are mainly applied under basic conditions. Phosphate and citrate are buffers that can be used over a broad pH range due to their multiple association constants. Borate exhibits very low conductivity compared to phosphate and other buffers. Buffer concentrations in the range of 10 mmol L^{-1} to approximately 100 mmol L^{-1} can be used. The electrolytes used should not possess any UV absorbance at low wavelengths.

An increase in ionic strength generates sharper peaks (zone focusing) due to the drop of the electrical field at the sample–electrolyte boundary and sample loading capacity can be increased. High ionic strengths induce high electrical currents and the increase of Joule heating can give rise to band broadening.

Dispersive effects caused by the interaction with the capillary wall are usually not a problem with peptides but larger species can exhibit characteristics similar to proteins in that they tend to adsorb at the capillary wall.

High ionic strength, extreme pH values and buffer additives competing in adsorption with the peptides are strategies of optimization which can be adapted from protein analysis. At extreme pH values, peptides and the capillary wall are equally charged so electrostatic repulsion diminishes adsorption. Coated capillaries have been used to suppress this phenomenon.

High salt content in the sample may destroy the separation efficiency of the electrophoretic system so sample preparation steps must remove the high ionic strength in the sample.

Enhancement in selectivity can be attained if an additional equilibrium is superimposed on to the electrophoretic process. Mostly the additives used for this are complexing agents which interact with specific groups of the peptide.

As for amino acids, metal ions can be employed for the separation of peptides and histidine-containing peptides especially interact with zinc salts. Separation of two histidine dipeptides (L-L, D-L) can be attributed to favourable steric arrangement of the histidine residues in one isomer.

Cyclodextrins form dynamic inclusion complexes with hydrophobic parts of the peptide, e.g. with amino acid residues containing aromatic rings like phenylalanine. The mass of a complexed analyte is

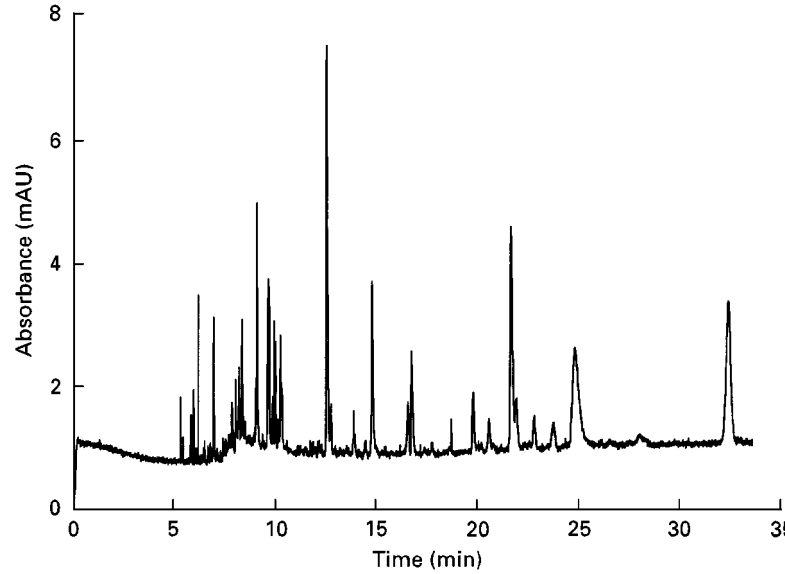

Figure 6 Tryptic digest of a haemoglobin variant separated by CZE. Capillary: poly(vinyl alcohol) coated fused silica capillary 50 μm i.d., 50/57 cm, buffer: phosphate 50 mmol L^{-1}; pH = 2.5; E = 526 V cm^{-1}, 214 nm; injection 0.5 psi, 5 s.

increased in this way and lower charge-to-mass ratio results in decreased mobility.

Ion-pairing reagents like short chain alkylsulfonic acids are particularly applied to adjust selectivity for hydrophobic peptides. Concentrations below the critical micellar concentration are used. The mechanism is based on the interaction between the hydrophobic surface of the peptides and the hydrophobic alkyl chain. Depending on the hydrophobicity of a peptide, different amounts of alkylsulfonic acid are attracted. Charge-to-mass ratios of the individual peptides are influenced to a different extent leading to the separation of the species.

A second approach to impart selectivity to large peptides with identical mobilities but different hydrophobicities is the use of ion-pairing reagents above their critical micellar concentration (CMC). This technique may also be used for peptides differing in neutral amino acids such as ala, val, leu or ile. MEKC takes advantage of the partitioning of the peptides between the electrolyte and the pseudo-stationary phase of the micelles. Hydrophilic moieties of the peptide interact with the outer polar sections of the micelle whereas hydrophobic parts are situated in the inner hydrophobic sphere. These peptide–micelle aggregates possess a different mobility compared to the electrophoretic mobility of the peptide in free solution.

Types of surfactants employed are divided into anionic, cationic and nonionic micelle-forming reagents. Because of the different charges, different migration directions are obtained. Negatively charged SDS, one of the most frequently used additives, migrates counter to the EOF and is used in concentrations up to approximately 150 mmol L^{-1}.

Common positively charged reagents are cetyl, dodecyl and hexadecyltrimethylammonium salts. These reagents invert the EOF at concentrations below the CMC so that as a consequence the polarity of the applied electrical field has to be reversed.

The addition of organic solvents such as methanol, ethanol, acetonitrile or tetrahydrofuran can provide selectivity for closely migrating peptides. These changes can be mainly attributed to solvation of side chains and variations in dissociation of the functional groups of the peptide. Additionally the EOF is modified due to altering the ζ- potential and the increase in buffer viscosity which generates a lower EOF and lower currents. In this way separations have been established for peptides differing in only a single neutral amino acid.

Peptides, especially large peptides with protein-like characteristics, sometimes tend to adsorb at the capillary wall. Beside the possibilities for avoiding dispersive effects mentioned above, the addition of amino- or diamino compounds like diamino-pentane, butane or morpholine can diminish the peptide–wall interaction. Competing equilibria in the electrostatic attraction between analyte-silanol and amine-silanol groups suppress the adsorption of the peptide. Another approach to reduce adsorption is derivatization of the silanol groups with an uncharged polymer (coated capillaries).

Detection Techniques

The detection of peptides suffers from the same difficulties as described for amino acids. Additionally only a few amino acids (phe, try, tyr and to a lesser extent his, arg, gln, asn) provide residues with strong chromophores.

Measuring UV absorbance at low wavelengths (< 220 nm) is the commonest mode of detection to give limits of detection of about 1 μg mL^{-1} ($\sim 10^{-5}$–10^{-6} mol L^{-1}) which are sufficient for most applications. Spectra obtained by a photodiode array detection support identification of impurities in peptide synthesis due mainly to the absence of the characteristic absorbance of aromatic residues at 220 nm (**Figure 7**).

Indirect techniques can be applied as for amino acids.

Detection of trace amounts of peptides requires more sensitive methods and sensitivity can be improved by fluorescence methods.

This approach faces the same difficulties as UV absorbance detection in that only try and, to a lesser extent, tyr and phe exhibit native fluorescence when excited at 280 nm (Xe-lamp). However, this 'natural specificity' facilitates selective identification of try-containing peptides. In addition, indirect fluorescence detection using salicylic acid for anionic charge peptides (basic buffers) or quinine for the positive mode (acidic buffer) have been applied.

To accomplish lower detection limits for a broader range of species derivatization techniques have to be applied and all the agents described for amino acids can be used for the derivatization of peptides.

Increased interest is being paid to mass spectrometric techniques for the characterization of peptides, especially soft ionization techniques like electron spray ionization (ESI). A promising approach towards nonfragmented peptides is the matrix-assisted laser desorption ionization with time-of-flight mass spectrometers (MALDI-TOF).

Concluding Remarks

CE has proved to be a versatile method for the high efficient separation of complex mixtures of amino

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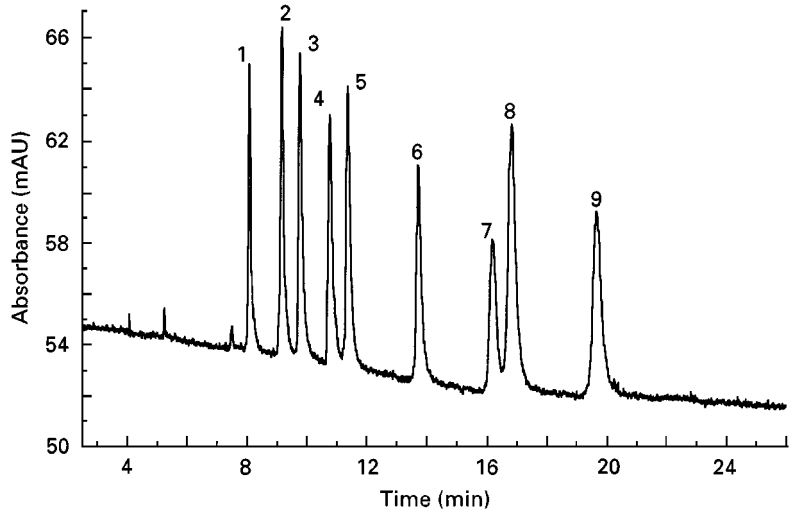

Figure 7 CZE separation of a peptide mixture. Capillary: ethylene/vinyl acetate dynamically coated with polyvinyl alcohol 75 μm i.d., 25/45 cm, buffer: phosphate 50 mmol L^{-1}; pH = 2.5; E = 155 V cm^{-1}, 200 nm; injection 50 mbar, 5 s. 1, Bradykinin; 2, angiotensin II; 3, α-MSH; 4, TRH; 5, LH-RH; 6, leucin enkephalin; 7, bombesin; 8, methionin; 9, oxytocin.

acids and peptides due to the manifold separation modes that can be applied. Short analysis times, easy manipulation of separation conditions and small injection volumes (nanolitres) are further advantages.

The field of biomedical and clinical amino acid and peptide analysis is still under investigation, especially as the transfer and adaptation of the separation modes to a broader range of real samples has to be established. Thus monitoring of *in vivo* processes, e.g. analysis of neurotransmitters in cerebrospinal fluid after online microdialysis, could be realized.

This is directly related to further improvements in reproducibility and detection strategies.

The most promising techniques that will fulfil the demands of trace analysis in biological fluids are CE-LIF and CE-MS.

Future trends are micro-fabricated CE devices implementing CE technology on a microchip and multiple capillary arrays allowing simultaneous analysis of up to 96 samples. Thus, a down-scaling of the analytical process and the performance of high throughput analysis could be achieved.

Further Reading

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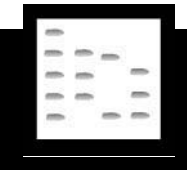
ANAESTHETIC MIXTURES: GAS CHROMATOGRAPHY



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Introduction

Today, anaesthetists normally use mixtures of nitrous oxide and oxygen as a background anaesthetic and carrier to introduce a potent volatile liquid